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INCREASED EXPRESSION OF THE *c-fos* PROTO-ONCOGENE IN BONE FROM PATIENTS WITH FIBROUS DYSPLASIA

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Abstract *Background.* Fibrous dysplasia is characterized by intense marrow fibrosis and increased rates of bone turnover. The lesions of fibrous dysplasia resemble those described in the long bones of transgenic mice overexpressing the c-*fos* proto-oncogene. Activating mutations in the α subunit of the stimulatory guanine-nucleotide–binding protein ($G_s\alpha$) linked to adenylate cyclase have recently been described in bone cells from patients with the McCune–Albright syndrome and fibrous dysplasia.

Methods. We used in situ hybridization to determine the level of expression of c-*fos* in bone-biopsy specimens from two normal subjects, eight patients with fibrous dysplasia, and six patients with other bone disorders characterized by high rates of bone turnover. The probe used corresponded to the fourth exon of the c-*fos* gene.

Results. High levels of c-*fos* expression were detected in the bone lesions from all eight patients with fibrous dysplasia. No expression of c-*fos* was detected in bone specimens from the normal subjects or from specimens of normal bone obtained from patients with fibrous dysplasia. The cells that expressed c-*fos* in the dysplastic lesions were fibroblastic and populated the marrow space. A very low level of c-*fos* expression was detected in the biopsy specimens from the patients with other bone diseases. One patient with polyostotic fibrous dysplasia and one patient with the McCune–Albright syndrome were tested for the previously described $G_s\alpha$ gene mutations and were found to express these mutations in bone.

Conclusions. Increased expression of the c-*fos* proto-oncogene, presumably a consequence of increased adenylate cyclase activity, may be important in the pathogenesis of the bone lesions in patients with fibrous dysplasia. (N Engl J Med 1995;332:1546-51.)

FIBROUS dysplasia is a sporadic developmental condition that affects bone structure.[1] The disease is characterized histologically by the persistence of woven bone where lamellar bone would normally develop. Marrow fibrosis is intense, and the rate of bone turnover is increased. The mixture of calcified cartilage and woven bone that makes up most of the bone accounts for its poor mechanical strength and the severe deformities that ensue.

Recently, mutations in the α subunit of the gene for the stimulatory guanine-nucleotide–binding protein ($G_s\alpha$) linked to adenylate cyclase have been described in bone cells from patients with the McCune–Albright syndrome[2-4] who have polyostotic fibrous dysplasia as a clinical manifestation of their disease (other manifestations are café au lait spots and various endocrinopathies). These mutations lead to the constitutive activation of adenylate cyclase.[5] The increased signaling through the cyclic AMP (cAMP) pathway is believed to be responsible for the clinical characteristics of the McCune–Albright syndrome, although the molecular events resulting from the constitutive production of cAMP have not been characterized.

The product of the c-*fos* proto-oncogene is a nuclear protein that forms heterodimers with the proteins encoded by the *jun* family of proto-oncogenes to form the transcription factor AP-1, which binds to specific sequences in the promoter region of target genes to modulate their expression.[6] Examination of mutations involving both gain of function and loss of function has demonstrated that bone is a physiologic target tissue of the action of c-*fos*.[7-9] In particular, transgenic mice overexpressing c-*fos* have abnormal bone remodeling characterized by bone marrow fibrosis and enhanced formation of woven bone.[9] These lesions closely resemble those that occur in patients with fibrous dysplasia. In this study, we used in situ hybridization to measure the expression of c-*fos* in bone-biopsy specimens from normal subjects and patients with fibrous dysplasia and found that the expression of c-*fos* messenger RNA (mRNA) was increased in bone lesions from the patients.

METHODS

Study Subjects

We studied biopsy specimens of iliac-crest bone from two healthy 8-year-old boys, eight patients with fibrous dysplasia (age range, 6 to 17 years), and six patients with other bone diseases (age range, 2 to 64 years). The biopsies were performed in the normal subjects and control patients during minor orthopedic procedures to build a reference data base for pediatric bone histomorphometry (unpublished data) and in the patients to confirm the diagnosis. In all patients with fibrous dysplasia, the biopsy specimens were obtained from areas of abnormal bone; in two patients with polyostotic fibrous dysplasia, we also obtained bone from the contralateral normal iliac crest (normal bone means histologically normal in this context). The lesions caused by fibrous dysplasia were histologically similar in all patients, whether they had monostotic or polyostotic disease, and were characterized by marrow fibrosis, trabeculae made of woven bone, high rates of bone turnover, and poorly organized cortexes.

Among the eight patients with fibrous dysplasia, one (a 14-year-old boy) had bone lesions involving the entire skeleton, a condition considered to be a discrete clinical entity — panostotic fibrous dysplasia[10]; two (a 10-year-old girl and a 7-year-old boy) had the McCune–Albright syndrome; three (a 17-year-old girl, an 11-year-old boy, and a 13-year-old boy) had polyostotic fibrous dysplasia; and two (a 6-year-old boy and a 15-year-old boy) had monostotic fibrous dysplasia. The disease was diagnosed in all patients after the occurrence of limb deformities or atraumatic fractures through a lesion. Among the six patients with other bone diseases, one (a 2-year-old

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girl) had hypocalcemic vitamin D–resistant rickets, one (a 64-year-old man) had Paget's disease, one (a 2-year-old girl) had the Proteus syndrome (hemihypertrophy, subcutaneous tumors, and macrodactyly), and three (a 5-year-old girl, a 13-year-old girl, and a 6-year-old boy) had osteogenesis imperfecta type IV. These patients were selected on the basis of variable degrees of marrow fibrosis and increased rates of turnover observed on biopsy.

The studies were approved by the appropriate institutional review committees, and informed consent was obtained from all subjects or their parents.

In Situ Hybridization

The biopsy specimens were prepared with the use of standard techniques for bone histomorphometry.[11] Undecalcified 6-μm sections were immersed four times in ethylene glycol monoethyl ether acetate for 10 minutes. After rehydration, the sections were denatured by incubation in 0.2 N hydrochloric acid for 20 minutes followed by incubation in 2× sodium citrate buffer (SSC) (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) at 70°C. The tissues were then fixed for five minutes in 4 percent paraformaldehyde at room temperature and blocked with dithiothreitol, iodoacetamide, and *N*-ethylmaleimide.[12] Hybridization was carried out at 42°C for 30 minutes in a hybridization mix.[12] The human c-*fos* riboprobe was generated by subcloning the *Nco*I-to-*Sca*I fragment of the human c-*fos* gene, which spans the fourth exon, into the pGEM-3Z vector (Promega, Madison, Wis.). The 71-base-pair (bp) antisense c-*fos* probe labeled with uridine 5′-α-[^{35}S]thiotriphosphate was then synthesized by in vitro transcription of the *Bsg*I-digested plasmid with T7 polymerase according to the manufacturer's instructions. The 73-bp control probe used to detect nonspecific hybridization was prepared by transcribing the *Sma*I-linearized pBluescript SK(–) phagemid (Stratagene, La Jolla, Calif.) with T7 polymerase. The c-*fos* probe contained 12 labeled uridine residues, whereas the control probe contained 15 labeled uridine residues. After hybridization, the sections were washed five times at 45°C for 12 minutes each time in 2× SSC and twice for 15 minutes in 0.2× SSC dehydrated in ethanol, and exposed to Kodak NTB-2 photographic emulsion (Eastman Kodak, Rochester, N.Y.).

Immunofluorescence Assay

Cryosections from the bone lesion from the 10-year-old girl with fibrous dysplasia were mounted on slides coated with poly-L-lysine and stained with monoclonal anti–c-*fos* antibody Ab-1 (Oncogene Science, Manhasset, N.Y.), as described previously.[13] The dilutions used were 1:5 for the primary antibody and 1:100 for the secondary antibody.

Restriction-Site Polymorphism

Total RNA was isolated[14] from cells from lesions caused by fibrous dysplasia in the 7-year-old boy with the McCune–Albright syndrome and the 14-year-old boy with panostotic fibrous dysplasia.[10] Cells were also obtained from normal bone (an iliac-crest–biopsy specimen from an eight-year-old boy) and grown according to published procedures.[15] The RNA was reverse-transcribed and amplified with the polymerase chain reaction (PCR) as described elsewhere.[16] The 5′ end-labeled upstream primer and the downstream primer were from exon 7 and exon 10, respectively.[16]

Results

Extensive c-*fos* hybridization signals were detected in samples of the bone lesions from all eight patients with fibrous dysplasia, and the level of c-*fos* expression was similar in all sections of abnormal bone from these patients. The cells in the lesions that expressed c-*fos* were the fibroblastic cells that populate the bone marrow space (Fig. 1A and 1C). All eight samples of bone lesions studied had substantially more signals than the samples of histologically normal bone obtained from two of the patients (Fig. 1E) or the bone samples from the two normal subjects. Few grains were seen when the sections were probed with an unrelated control probe under identical conditions (Fig. 1B, 1D, and 1F).

To ascertain that the expression of the c-*fos* protein correlated with the expression of the c-*fos* mRNA, we immunostained the sections from one patient with fibrous dysplasia with an anti–c-*fos* monoclonal antibody. We did not immunostain histologically normal bone because of the very low level of expression of c-*fos* mRNA in these samples. The c-*fos* protein was detected in the same fibroblastic cells that overexpressed c-*fos* mRNA within the marrow cavity of the fibrous dysplasia lesion (data not shown). The c-*fos*–protein signal was specific, on the basis of control staining reactions.

Only the cells in the bone lesions from patients with fibrous dysplasia showed increased expression of c-*fos* mRNA (Fig. 2A and 2B). The level of c-*fos* mRNA was not increased in the bone specimen from a patient with hypocalcemic vitamin D–resistant rickets (Fig. 2C and 2D). The specimen from a patient with Paget's disease showed no c-*fos* hybridization signal when the exposure time was short (Fig. 2E and 2F). Longer exposure to the photographic emulsion allowed detection of c-*fos* mRNA in this biopsy specimen, as recently reported[17] (data not shown). A very low level of c-*fos* expression was detected in the biopsy specimen from a patient with the Proteus syndrome (Fig. 2G and 2H), and in regions of fibrous tissue in specimens from three patients with osteogenesis imperfecta type IV (data not shown).

Replacement of the arginine residue at position 201 of the $G_s\alpha$ gene subunit by either histidine or cysteine results in an abnormal $G_s\alpha$ protein that stimulates adenylate cyclase in a constitutive fashion.[5] These mutations have been described in bone cells obtained from the lesions caused by fibrous dysplasia and from other affected tissues in patients with the McCune–Albright syndrome.[2-4] We assessed whether activating $G_s\alpha$ mutations were present in one patient with the McCune–Albright syndrome and one patient with panostotic fibrous dysplasia.[10] The missense mutation causing the substitution of histidine for arginine at position 201 introduces a novel cleavage site for the *Nla*III restriction endonuclease.[18] Reverse-transcribed RNA from cultured bone cells from lesions in these two patients and from a normal subject was amplified by PCR with labeled primers flanking the mutation site, digested with *Nla*III, and analyzed by polyacrylamide-gel electrophoresis. Figure 3 shows the diagnostic *Nla*III restriction fragment detected in one of the patients (lane 3), confirming the Arg-to-His mutation. The other patient did not have this mutation (Fig. 3, lane 2), but was confirmed to express the Arg-to-Cys mutation on the basis of the hybridization of reverse-transcribed RNA with allele-specific oligonucleotide probes[16] (data not shown). Samples from a normal subject tested negative for both mutations in all assays (Fig. 3, lane 1; data not shown). The $G_s\alpha$

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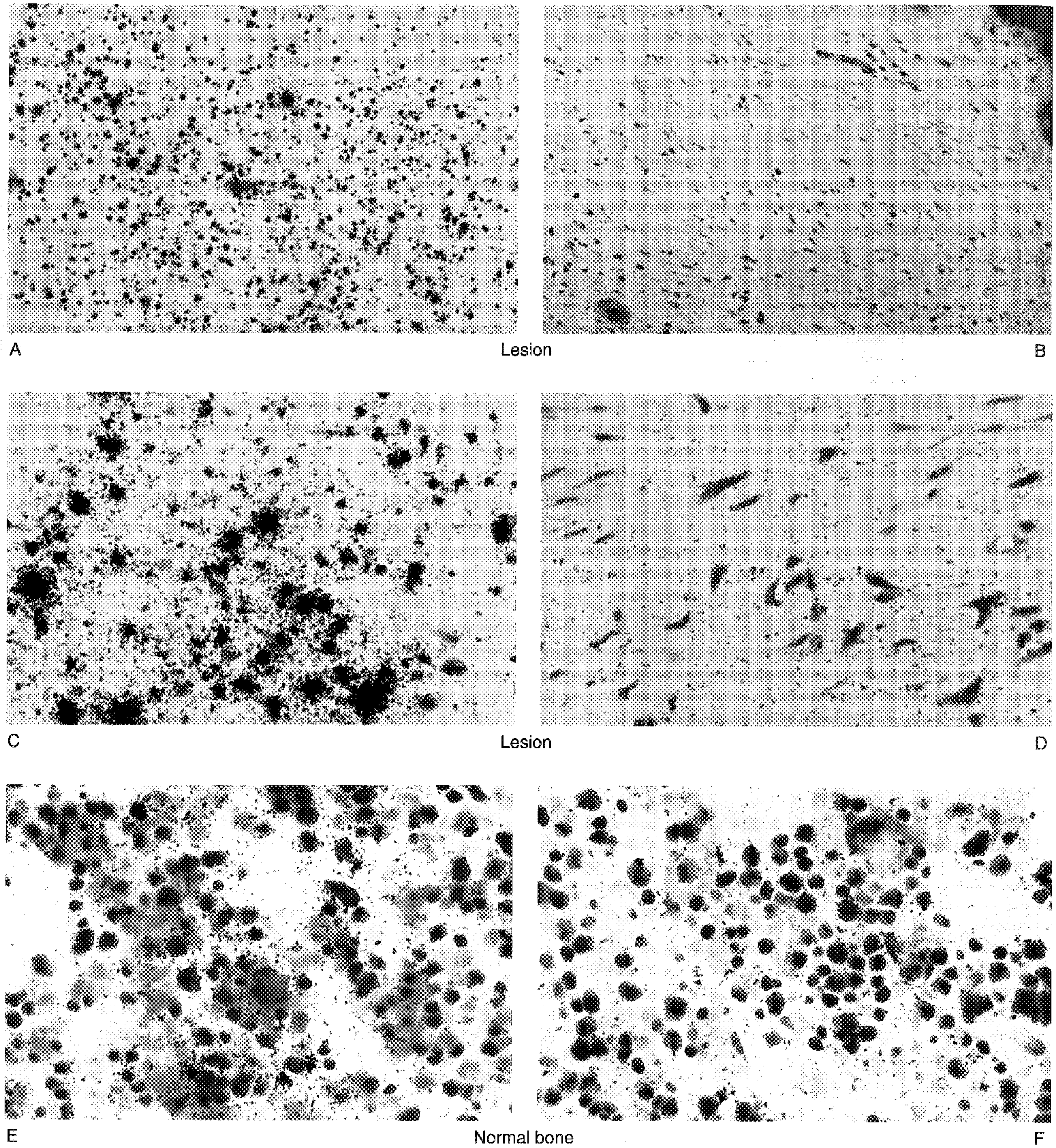
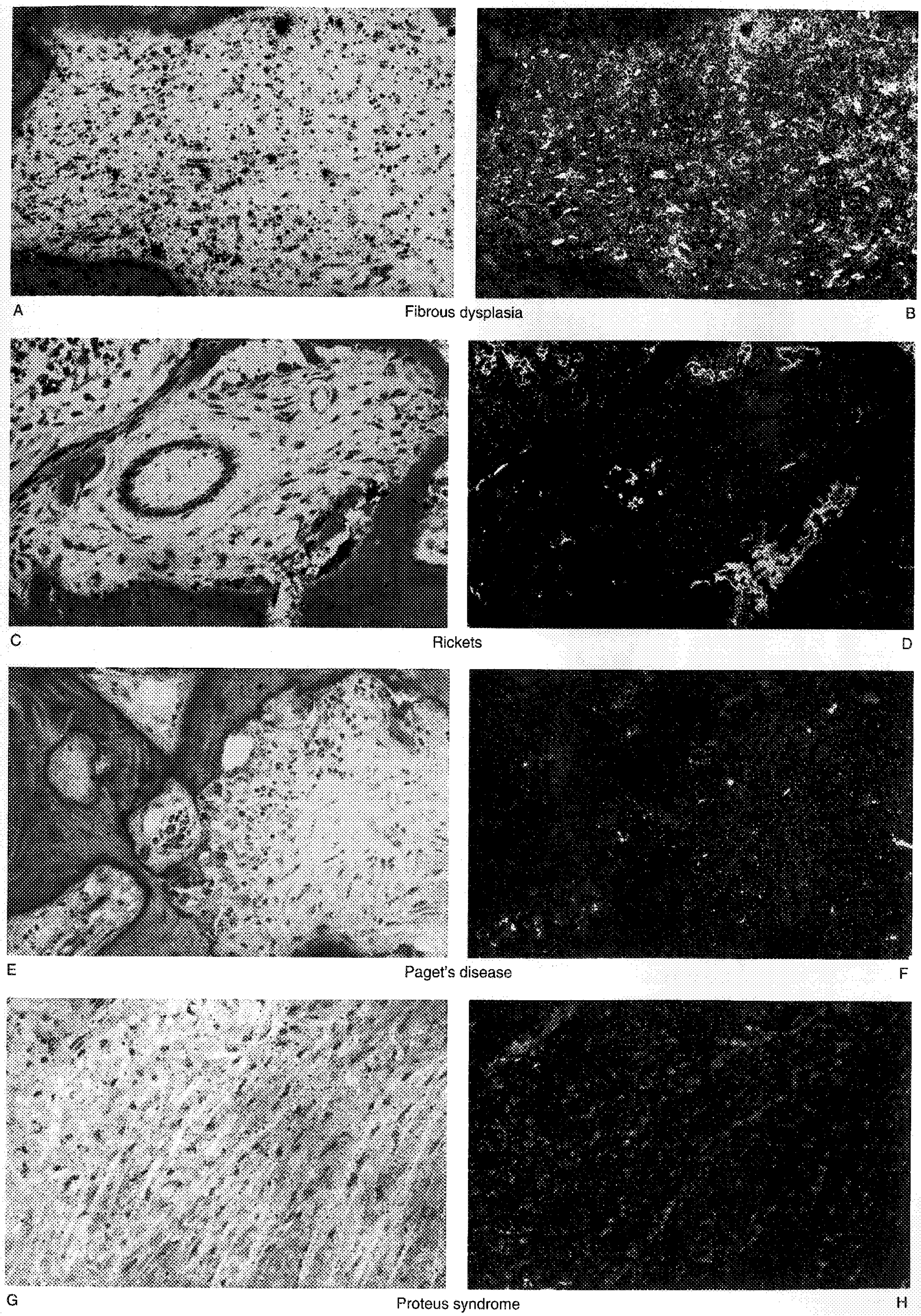


Figure 1. Expression of c-*fos* mRNA in Biopsy Specimens of Lesions and Normal Bone from a Patient with Polyostotic Fibrous Dysplasia.

The bright-field photomicrographs were taken at low power (×740; Panels A and B) and high power (×2300; Panels C, D, E, and F). The sections were hybridized with either the specific c-*fos* probe (Panels A, C, and E) or a control probe (Panels B, D, and F) under identical conditions. After in situ hybridization and autoradiography, the slides were counterstained with toluidine blue. Note the extensive c-*fos* hybridization signal in the lesion (Panels A and C), which is absent in histologically normal bone (Panel E).

Figure 2 (Facing Page). Expression of c-*fos* mRNA in Biopsy Specimens of Lesions from a Patient with Fibrous Dysplasia (Panels A and B) and in Bone Specimens from Patients with Hypocalcemic Vitamin D–Resistant Rickets (Panels C and D), Paget's Disease (Panels E and F), or the Proteus Syndrome (Panels G and H).

Panels A, C, E, and G are bright-field photomicrographs and Panels B, D, F, and H are darkfield photomicrographs of sections of bone probed with the specific c-*fos* riboprobe. Increased c-*fos* expression was detected only in the biopsy specimen from the patient with fibrous dysplasia. The specimens were counterstained with toluidine blue (×740).



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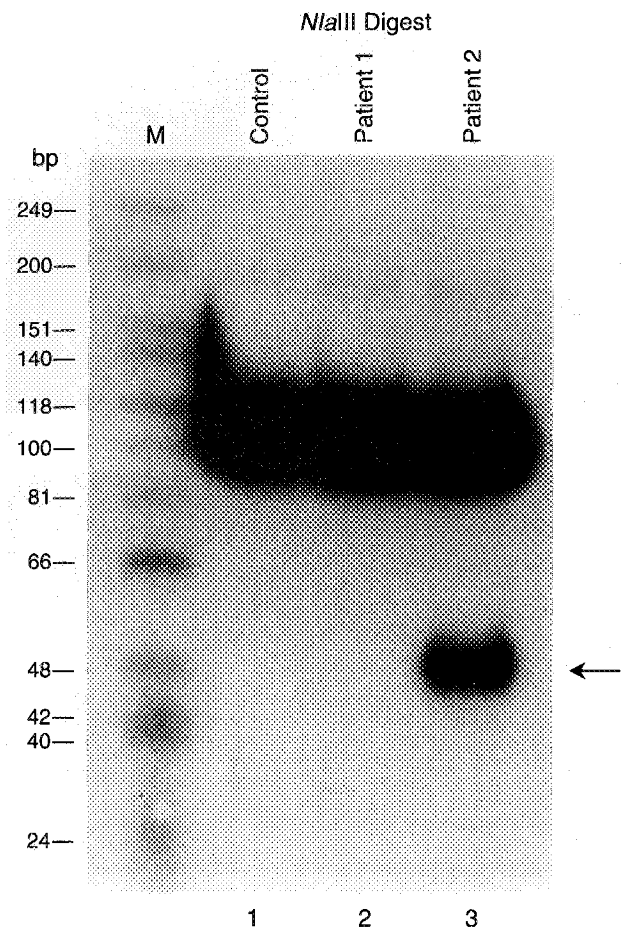

Figure 3. Analysis of Restriction-Site Polymorphisms in the $G_s\alpha$ Gene in One Patient with Fibrous Dysplasia (Patient 1), One Patient with the McCune–Albright Syndrome (Patient 2), and a Normal Subject.

Reverse-transcribed RNA from a normal subject (lane 1) and two patients (lanes 2 and 3) was amplified by PCR with labeled primers flanking exon 8 of the $G_s\alpha$ gene, digested with *Nla*III, and analyzed on a 15 percent polyacrylamide gel. The arrow shows the diagnostic *Nla*III restriction fragment introduced by the missense mutation resulting in the substitution of histidine for arginine.[18] M denotes the molecular size markers.

gene was not studied in any tissue from any of the other patients.

Discussion

We found that the expression of c-*fos* mRNA and protein was increased in bone lesions from eight patients with fibrous dysplasia, at least two of whom had activating mutations of the $G_s\alpha$ gene in bone cells. The increase in c-*fos* mRNA appears to be specific for this bone disease and not linked to a phenotype of a high rate of bone turnover or to fibrotic tissue in general. These results support a role for c-*fos* in fibrous dysplasia.

In patients with Paget's disease c-*fos* is expressed in osteoclasts,[17] but only in cells attached to the bone surface and not in cells from the marrow cavity.[17] In our study, no c-*fos* signal was detected in the biopsy specimen from a patient with Paget's disease. We were able to detect c-*fos* mRNA in bone sections from this patient when longer exposure times were used, thus confirming the published report.[17] The expression of c-*fos* was not increased in the biopsy specimens from patients with hypocalcemic vitamin D–resistant rickets, the Proteus syndrome, or osteogenesis imperfecta type IV. Thus, our results suggest that overexpression of c-*fos* in fibrotic cells from bone lesions is specific for fibrous dysplasia.

Transgenic mice overexpressing c-*fos* have abnormal bone remodeling,[9] and osteosarcomas develop in some.[19] The tissue content of c-*fos* protein is also increased in human osteosarcomas.[20] Osteosarcomas eventually develop in about 0.5 percent of patients with fibrous dysplasia and 4 percent of patients with the McCune–Albright syndrome.[21] The increased expression of c-*fos* mRNA in lesions caused by fibrous dysplasia suggests that the overexpression of c-*fos* may represent the first step in the multistep carcinogenesis of bone sarcomas.[22]

A number of recent reports have linked mutations in both G protein–coupled receptors and G proteins to disease phenotypes,[23] but the molecular targets of the activated signal-transduction pathways remain to be identified. The accumulation of intracellular cAMP activates protein kinase A to phosphorylate specific protein substrates and leads to the regulation of gene transcription by modulating the phosphorylation state of cAMP-response-element–binding protein members of the family of transcription factors.[24] These nuclear proteins bind specific sequences, termed "cAMP response elements," in the promoter regions of target genes to modulate their transcription.[25] The human c-*fos* promoter contains a functional cAMP response element.[26] The increased production of cAMP in bone cells with the $G_s\alpha$ mutations most likely leads to elevated c-*fos* expression through the c-*fos* cAMP response element. Indeed, expression of an exogenous activated $G_s\alpha$ protein leads to increased transcription of c-*fos*.[27]

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